CARDIOPULMONARY DYSFUNCTIONS CAUSED BY INTRAVENOUS COLLAGEN-INDUCED RELEASE OF ADP AND ATP FROM PLATELETS

ITSURO KOBAYASHI* AND PAUL DIDSHEIM**

Bovine tendon collagen suspension (4.5 mg/kg) was injected rapidly into the femoral vein of 14 normal (untreated) and 8 busulfan treated rats. Transient effects included decreased platelet counts and arterial PO₂, increased central venous pressure, apnea, bradycardia and variable A-V block. These findings were most prominent within 1 minute after injection and subsided or disappeared by 10 minutes. During this period, ADP and ATP in platelet-free plasma from carotid arterial blood were measured in a liquid scintillation counter using the firefly luciferase assay. In normal (untreated) rats, collagen injection was followed by increases in plasma ADP and ATP, a rise in plasma hemoglobin and minimal changes of fibrinogen and hematcrit. Pathological observations indicated the platelet emboli in pulmonary vessels. In contrast, rats made thrombocytopenic by intraperitoneally injected busulfan prior to collagen injection had minimal or no change in platelet count, plasma ADP, ATP, hemoglobin, fibrinogen, or cardiopulmonary functions following collagen injection. These findings suggest that collagen injection causes release of ADP and ATP from platelets; released ADP induces platelets to form aggregates which lodge in the coronary and pulmonary microcirculations and elsewhere, resulting in thrombocytopenia and the cardiopulmonary dysfunction, in the presence of shear, of red cells with vessel surfaces altered by platelet aggregates.

Key Words:
Platelet aggregate
Collagen
ADP
ATP
Cardiopulmonary dysfunctions
A-V block

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* The 3rd Department of Internal Medicine, Tokyo Medical and Dental University, School of Medicine, Yushima, Bunkyo-ku, Tokyo, Japan
** Thrombosis Research Laboratory, Section of Laboratory Hematology, Department of Laboratory Medicine, Mayo Clinic, Rochester, Minnesota, U.S.A.

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circulation induced by ADP caused these changes. So we tried to observe effects of intravenous injection of collagen on plasma levels of adenine nucleotides, fibrinogen and hemoglobin and on cardiopulmonary functions.

MATERIALS AND METHODS

Twenty-two male 400 – 600 g Wistar rats were anesthetized with pentobarbital. A polyethylene tracheotomy tube was inserted. The right carotid artery and jugular vein were cannulated with polyethylene cannula (Becton Dickinson) for collection of arterial blood sample and measurement of central venous pressure (CVP). The right femoral vein was cannulated for injection of collagen (Fig. 1). Heparin, 100 Units/kg, was injected to prevent clotting in the cannulas during the experiment. Blood for platelet counts, white blood cell counts (WBC) and gas analysis was taken from carotid artery cannula into a plastic syringe containing 2% EDTA-tris buffer adjusted to pH 7.35. WBC and platelet count were counted on Coulter Model A and B counter. Blood gases were measured on an IL 112 blood gas analyzer. Subcutaneous needle electrodes for electrocardiogram (ECG) were applied on the chest and limbs. Respirations were

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**Firefly luciferase assay**

\[
\begin{align*}
\text{ATP: } y &= 10.812 - 4.261x + 0.426x^2 \\
\text{ADP: } y &= 24.464 - 7.911x + 0.660x^2
\end{align*}
\]

**Fig. 2.** ADP and ATP standard curve by measuring of firefly luciferase assay.
TABLE I  EFFECT OF RAPID INJECTION OF COLLAGEN (4.5 mg/kg) ON THE PLATELET AND WHITE BLOOD CELL (WBC) COUNT IN RAT

<table>
<thead>
<tr>
<th>No. Rats</th>
<th>Treatment</th>
<th>Preinjection Platelets, WBC</th>
<th>Platelets and WBC, in % of preinjection value</th>
<th>Minute(s) after collagen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>851±41 (x10³/cmm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10,500±1,100 (cmm)</td>
<td>31.2±4.0</td>
<td>67.9±6.1</td>
<td>93.7±5.2</td>
<td>102.8±2.0</td>
</tr>
<tr>
<td>14</td>
<td>Cytopenic</td>
<td>303±42 (1)</td>
<td>62.4±6.1*</td>
<td>75.3±4.0*</td>
</tr>
<tr>
<td>11,700±400 (1)</td>
<td>71.6±7.8*</td>
<td>68.6±8.2</td>
<td>61.8±9.8*</td>
<td>64.4±13.3*</td>
</tr>
</tbody>
</table>

(1): 7-10 days after initiation of busulfan therapy
* : Compared to the value in nontreated rats (p < 0.01)

Changes induced by collagen (4.5mg/kg i.v.)

Fig.3. Changes induced by collagen (4.5 mg/kg i.v.). Effects of rapid intravenous injection of bovine tendon suspension (collagen) on cardiorespiratory functions and platelet and white blood cell count.

sensed by a temperature-sensitive transducer attached to the tracheotomy tube. ECG and respirations were recorded on a Grass Model

III D 6 channels electroencephalograph. CVP was adjusted at O level before the collagen injection. Fourteen untreated and 8 busulfan-treated rats
TABLE II  COLLAGEN-INDUCED (4.5 mg/kg) EFFECTS ON

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Arterial PO₂ in % of preinjection value</th>
<th>Rise in CVP (mmHg)</th>
<th>Heart rate in % of preinjection value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>3</td>
<td>10**</td>
</tr>
<tr>
<td>None</td>
<td>64.6 ±5.2</td>
<td>64.9 ±9.6</td>
<td>68.7 ±7.0</td>
</tr>
<tr>
<td>Cytopenic</td>
<td>87.0** ±12.6</td>
<td>97.3* ±2.7</td>
<td>99.7* ±6.6</td>
</tr>
</tbody>
</table>

++: Minute(s) after collagen (4.5 mg/kg)
*: Compared to the value in untreated rats (p < 0.01)
**: Compared to the above (p < 0.05)

Collagen

V₁

72 sec interval

---

1 sec

Fig. 4. Effects of collagen injection (3.0 mg/kg i.v.) on electrocardiogram.

were injected with 4.5 mg/kg of collagen intravenously within one second’s time. Busulfan (Burroughs, Wellcome) was suspended in polyethylene glycol 400 (Fisher) in a concentration of 10 mg/ml. Thrombocytopenia and leukopenia were induced by administering busulfan, 25 mg/kg intraperitoneal injection, in two doses three days apart. WBC and platelet count were measured before the agents and 3, 7, and 10 days thereafter. The above rats were killed after the experiment and the organs (especially lungs) were observed histologically to find platelets embolizations.

ADP and ATP were measured by the firefly luciferase method of Holmsen et al. using a Packard Liquid Scintillation counter Model 4322 to measure emitted light. ADP and ATP standards were serially diluted with glass distilled water to desired moles (0.9, 1.8, 4.5 and 9.0 x 10⁻¹² moles for ATP standards, 1.1, 2.1, 5.3 and 10.5 x 10⁻¹² moles for ADP standards). Blood (9 volumes to 1 volume 2% Na₂ EDTA)
THE CARDIOVASCULAR SYSTEM

<table>
<thead>
<tr>
<th>No.</th>
<th>Duration</th>
<th>Resp. arrest</th>
<th>Resp. rate in % of preinjection value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No.</td>
<td>Duration</td>
</tr>
<tr>
<td></td>
<td>rats</td>
<td>sec.</td>
<td>sec.</td>
</tr>
<tr>
<td>14/14</td>
<td>127.1</td>
<td>14/14</td>
<td>23.3</td>
</tr>
<tr>
<td>±16.9</td>
<td>±3.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0/8*</td>
<td>0*</td>
<td>0/8*</td>
<td>0*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TABLE III  INCREASE OF ATP AND ADP AFTER INJECTION OF COLLAGEN (4.5 mg/kg)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ADP (μM)</th>
<th>ATP (μM)</th>
<th>Increase of ATP and ADP (Minute(s) after collagen)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>None</td>
<td>0.19±0.06*</td>
<td>0.20±0.05*</td>
<td>0.19±0.06*</td>
</tr>
<tr>
<td>Cytopenic</td>
<td>0.02±0.05</td>
<td>0.02±0.04</td>
<td>0.02±0.06</td>
</tr>
<tr>
<td></td>
<td>-0.03±0.02</td>
<td>0.04±0.06</td>
<td>0.02±0.03</td>
</tr>
</tbody>
</table>

* : Compared to the value in cytopenic rat (p < 0.01)

TABLE IV  ATP/ADP RATIO AFTER INJECTION OF COLLAGEN (4.5 mg/kg)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Minute(s) after collagen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Normal</td>
<td>1.2</td>
</tr>
<tr>
<td>Cytopenic</td>
<td>1.4</td>
</tr>
</tbody>
</table>

was collected from the carotid artery cannula. Plasma was prepared from whole blood sample by centrifuging 2,000 g 4°C for 15 minutes. One volume plasma was then transferred to 1 volume 96% ethanol and mixed. These mixtures were frozen and stored at -20°C for up to one month without significant effect for measurement. These mixtures were then thawed at 4°C, centrifuged at 2,000 g for 15 minutes to remove precipitated protein, and portions of the supernatant sampled for assay. For ADP assay, 20 μl of plasma-ethanol supernatant or a known amount of ADP were pipetted into 3 ml aliquots of activated PEP-PK (non heated KC1-MgSO4 phosphoenolpyruvate-pyruvate kinase, Sigma) solution for conversion of ADP to ATP. For ATP assay, 20 μl plasma-ethanol supernatant or a known amount of ATP were pipetted into 3 ml aliquots of inactivated PEP-PK (heated KC1-MgSO4 PEP-PK) solution. For both assays, sample vials were incubated for 6 minutes in a 37°C water bath, then vials were transferred to 100°C water bath and heated for 6 minutes to stop the reaction. Reaction mixture (luciferin-luciferase, Dupont bioluminescence kit) was injected into each sample. The sample was inverted, shaken gently and replaced into the counter, 20 seconds following injection, the vial was lowered into the counting chamber. The initial one count (10 seconds) was recorded.

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### TABLE V  EFFECT OF INJECTION OF COLLAGEN (4.5 mg/kg) ON THE PLASMA HEMOGLOBIN (Hb)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1</th>
<th>3</th>
<th>10</th>
<th>20</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>28.4±8.6*</td>
<td>39.0±8.9*</td>
<td>43.0±6.0*</td>
<td>37.6±12.2*</td>
<td>38.2±9.4*</td>
</tr>
<tr>
<td>Cytopenic</td>
<td>-0.4±1.3</td>
<td>3.0±5.8</td>
<td>-0.7±3.2</td>
<td>-1.6±2.9</td>
<td>-1.7±5.4</td>
</tr>
</tbody>
</table>

* : Compared to the value in cytopenic rat (p < 0.01)

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Plasma hemoglobin was measured by Crosby and Furth method. Twenty μl of standards and plasma were pipetted into cuvettes. One ml of benzidine and 1 ml of H₂O₂ were added and mixture was shaken well. Ten ml glacial acetic acid was added to mixture. The mixture was read at 515 millimicrons against a blank (benzidine – H₂O₂ – glacial acetic acid). Plasma fibrinogen was measured by Mann et. al. method. Thrombin was added to plasma to convert the fibrinogen to fibrin, which was then washed and measured colorimetrically by the biuret reaction.

Three g bovine tendon collagen (Sigma) was blended with 150 ml 0.9% NaCl for one and half four at high speed (Virtis 45, Gardiner, N.Y.) in an ice bath. The mixture was centrifuged at 1,000 g, 0°C, for 20 minutes and the supernatant stored in aliquots at -20°C until used. The amount injected was determined from the dry weight minus the weight of the contained NaCl.

Data in figures and tables are means and standard errors of 8 and 14 experiments in each group. Statistical significance was determined by Student’s t-test.

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RESULTS

ATP Standards and Conversion of ADP Into ATP by the PEP-PK System

The amount of ATP and ADP transformed by the PEP-PK system can be compared by measuring the counts of the light by the same preparation of firefly luciferase enzyme (FLE). ATP standards were tested eleven times in each mole. Correlation between moles of ATP (Y) and the counts (X) was \( Y = 10.812 - 4.261X + 0.426X^2 \) (X=ln(X), r=0.9999). Correlation between moles (Y) and the counts (X) was \( Y = 24.464 - 7.911X + 0.660X^2 \) (X=ln(X), r=0.9998). The two curves are almost superimposable, indicating complete conversion of ADP to ATP by PEP–PK in this ADP assay procedure (Fig. 2).

Effect of I.V. Injection of Collagen in Untreated Rat

Rapid I.V. injection of collagen (4.5 mg/kg) caused a prompt drop in platelet and WBC count to 20.4 ± 2.1% and 31.2 ± 4.0% of the preinjection value respectively one minute after the injection in 14 rats (Table I, Fig. 3). At the same time, arterial PO₂ decreased to 64.6 ± 5.2% of the preinjection value as shown in Table II. CVP increased in 4.0 ± 0.4 mmHg. Heart rate decreased to 63.0 ± 4.4% of the preinjection value accompanied with an appearance of sinus bradycardia and variable A–V block (Fig. 4). The A–V block and the sinus bradycardia appeared in all 14 cases and continued 4.8 to 258.8 seconds showing 127.1 ± 16.9 seconds in average. The rapid I.V. injection of collagen also had various respiratory effects including shallow respiration, slow respiration or respiratory arrest. The respiratory arrest appeared in all 14 cases and continued 10.2 to 29.8 seconds showing 23.2 ± 3.7 seconds in average. An average of respiratory rate at one minute after the collagen injection was 54.9 ± 4.6% of the preinjection value. The above changes showed a tendency to recovery within 10 minutes after the injection. Platelet and WBC count showed 27.5 ± 3.6% and 67.9 ± 6.1 respectively at 3 minutes, 52.8 ± 6.3% and 93.7 ± 5.2% at 10 minutes, 61.0 ± 6.4% and 102.8 ± 2.0% at 20 minutes, and 63.1 ± 5.4% and 103.3 ± 3.4% of the preinjection value at 30 minutes after the injection. At 10 minutes after the injection, the arterial PO₂ showed still decrease as 68.7 ± 7.0% of preinjection value, but all other parameters of cardiopulmonary functions did not show any statistically significant difference compared to the value in busulfan treated rats. The amount of ADP and ATP level in plasma increased after collagen (4.5 mg/kg) injection. ADP and ATP levels in plasma (Table III) increased 0.19 ± 0.06 μM and 0.20 ± 0.05 μM respectively at one minute, 0.25 ± 0.09 μM and 0.36 ± 0.10 μM at 3 minutes, 0.25 ± 0.09 μM and 0.32 ± 0.10 μM at 10 minutes, 0.20 ± 0.09 μM and 0.24 ± 0.08 μM at 20 minutes and, 0.18 ± 0.06 μM and 0.19 ± 0.08 μM at 30 minutes after collagen. The ratio of ATP/ADP was 1.0 to 1.2 (Table IV). Hemoglobin level in plasma increased also 28.4 ± 8.6 mg/100 ml at one minute, 39.0 ± 8.9 mg/100 ml at 3 minutes, 43.0 ± 6.0 mg/100 ml at 10 minutes, 37.6 ± 12.2 mg/100 ml at 20 minutes and 38.2 ± 9.4 mg/100 ml at 30 minutes after collagen injection (Table V). Fibrinogen and hemacrit did not change at 1, 3, 10, 20 and 30 minutes compared the value to cytopenic rat after collagen injection (Table VI). Patho-histological observations showed the lodging of platelet aggregates and did not show any fibrin deposits in pulmonary arterioles following collagen injection (Fig. 5).

Effect of I.V. Injection of Collagen in Busulfan Treated Rat

Before the treatment, their WBC was 10,300 ± 1,100 per cmm, hemacrit was 45.8 ± 1.5% and platelet count was 838,000 ± 36,000 per cmm. After 7–10 days of the treatment WBC was 1,700 ± 400 per cmm, hemacrit was 41.4 ± 1.3% and platelet count was 303,000 ± 42,000 per cmm. When these 8 rats were injected with collagen (4.5 mg/kg), the platelet count had returned pre-injection levels by 3 minutes whereas the untreated rats still had reduced levels at 30 minutes (Table I, Fig. 6).

In contrast to the untreated rats, this group of animals experienced no anepoa, arrhythmia, rise in CVP, arterial oxygen desaturation and no reduction in respiratory and heart rates (Fig. 6). These value showed a statistically significant difference (p<0.01–0.05) compared to each of the value in the untreated rat (Table II). The increase amount of ADP and ATP level in plasma were 0.02 ± 0.05 μM and −0.03 ± 0.02 μM respectively at one minute, 0.02 ± 0.04 μM and 0.04 ± 0.06 μM at 3 minutes, 0.02 ± 0.03 μM and 0.04 ± 0.02 μM at 10 minutes, 0.03 ± 0.06 μM and 0.02 ± 0.03 μM at 20 minutes, and 0.02 ± 0.06 μM and −0.14 ± 0.03 μM at 30 minutes (Table III). These values showed a statisti-
TABLE VI  EFFECT OF INJECTION OF COLLAGEN (4.5 mg/kg) ON THE FIBRINOGEN AND HEMATOCRIT

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Preinjection value</th>
<th>Changes in % preinjection value</th>
<th>Minute(s) after collagen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>None</td>
<td>Fibrinogen</td>
<td>294.6±21.6 (mg/100ml)</td>
<td>85.6±3.5</td>
</tr>
<tr>
<td></td>
<td>Hematocrit (%)</td>
<td>47.1±1.1</td>
<td>96.2±1.3</td>
</tr>
<tr>
<td>Cytopenic</td>
<td>Fibrinogen</td>
<td>236.6±32.3 (1)</td>
<td>87.1±7.4</td>
</tr>
<tr>
<td></td>
<td>Hematocrit (%)</td>
<td>41.4±1.3 (1)</td>
<td>95.7±4.1</td>
</tr>
</tbody>
</table>

(1): 7–10 days after initiation of busulfan therapy

Changes induced by collagen (4.5 mg/kg i.v.)
Thrombocytopenic rat (busulfan 25 mg/kg i.p. x 2)

Fig.6. Effects of collagen injection (4.5 mg/kg) on cardiopulmonary functions, platelet and white counts in thrombocytopenic rats previously treat with busulfan (25 mg/kg i.p. x 2).

cally significant difference compared to each of the untreated values (p<0.01). The ratio of ATP/ADP was 1.4 to 1.6 (Table IV). The in-
crease amount of plasma hemoglobin were −0.4 ± 1.3 mg/100 ml, 3.0 ± 5.8 mg/100 ml, −0.7 ± 3.2 mg/100 ml, −1.6 ± 2.9 mg/100 ml,
-1.7 ± 5.4 mg/100 ml at 1, 3, 10, 20 and 30 minutes respectively (Table V). These values showed statistically significant difference compared to each of the untreated values (p<0.01). The changes of plasma fibrinogen and hematcrit were the same pattern in comparison to the untreated rat (Table VI).

Discussion

When collagen (4.5 mg/kg) was injected intravenously into rat, the changes of platelet count and cardiopulmonary dysfunctions such as thrombocytopenia, apnea, rise in CVP, fall in arterial PO2 and A-V block were occurred (Fig. 3, 4). We have demonstrated that the changes in platelet count and cardiopulmonary dysfunctions that follow ADP and collagen injection are produced by platelet aggregates which lodge transiently in the coronary, pulmonary and other microcirculatory beds\textsuperscript{2}–\textsuperscript{4}. The effects occurred immediately after ADP injection; in contrast, after collagen injection they follow injection only after a lag period.\textsuperscript{2} The difference in response of the time lag to ADP between collagen is of interest.

The rise in CVP and fall in arterial PO2 following collagen injection may be due to the transient lodging of platelet aggregates in pulmonary arteries. Pathological changes showed the platelet thrombi in pulmonary vessels following collagen injection. The heart block (Fig. 4, Table II) may be due to the transient lodging of platelet aggregates in the coronary arterioles in the vicinity of A-V nodal tissue. Perhaps contact of collagen with platelets\textsuperscript{10} in the systemic circulation leads to release of ADP which then circulates through the pulmonary circuit and thence to the coronary circulation. In fact, increase amount of ADP (0.19–0.25 μM) after collagen injection is approximate amount of 30–40 μg/kg injection of ADP. This amount of ADP showed the falling platelet count and cardiopulmonary dysfunctions.\textsuperscript{2} In contrast to untreated rat, there were no release of ATP and ADP in plasma (Table III) and minimal drop of platelet count and cardiopulmonary changes (Table II, Fig. 6) in cytopenic rat.

Brain et al.\textsuperscript{11} produced the intravascular hemolysis with thrombin and endotoxin. Hemolysis in the small blood vessels at the site of thrombus formation is one possible explanation of this phenomena. Red cells may become temporarily attached to and disturbed by, the normal vascular endothelium. Such a mechanism might result in the localized attachment of the red cell membrane to fibrin present on the endothelial surface, with rupture of the membrane of red cells, release of hemoglobin and possibly the formation of red cell fragments and small spherocytes. Fibrin deposition was very severe and there occurred high hemoglobinemia. In contrast, our observations were minimal deposition of fibrinogen, severe drop in platelet counts and high to moderate hemoglobinemia. The shape changes of red cells by smears (Wright’s stain) were sometimes observed the presence of crenated and distorted cells, fragments and microspherocytes between 1 and 30 minutes after injection of collagen. These phenomena suggest embolizations of platelet aggregates in the small vessels induced by collagen caused hemoglobinemia.

Erythrocytes have been considered to be the source of the ADP and ATP.\textsuperscript{12} If all ADP and ATP in plasma after collagen injection were from distorted erythrocytes, the ratio of ATP/ADP should be 4–6.\textsuperscript{13} However, the ATP/ADP ratio observed in rat plasma following collagen injection was 1.0–1.6. These date suggest that most of ATP and ADP recovered from plasma in these experiments originated not from erythrocytes but from platelets, presumably from their nonmetabolic storage pool.\textsuperscript{14}

The suppressed cardiorespiratory and hemotologic response to collagen in rats pretreated with busulfan may be explained in a few ways; a) busulfan treatment may have left insufficient numbers of platelets to form clinically significant aggregates in the microcirculation after collagen injection; b) busulfan treatment, by blocking further production of platelets, may have shifted the mean age of the remaining circulating platelets toward senescent ones, less responsive to collagen’s aggregating stimulus. It is of interest that, despite the fact that the platelet count following busulfan treatment was no lower 40% of baseline, no detectable ADP or ATP was released into the plasma. These findings suggest that the ADP and ATP stores of the remaining circulating platelets may be reduced or unavailable for release.

Clinically, pathogenesis of thrombotic disorders such as myocardial infarction and cerebral thrombosis are considered interaction of circulating platelets with subendothelial collagen at sites of endothelial discontinuity. So it is important to know the early stages of thrombogenesis and to prevent these events and it may
have correlation with treatment.

Acknowledgments
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REFERENCES